

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

PF95PCTSEQ/dln

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/936677

INTERNATIONAL APPLICATION NO.

PCT/FR00/00623

INTERNATIONAL FILING DATE

15 MAR 2000 (12.03.00)

PRIORITY DATE CLAIMED

15 MAR 1999 (15.03.99)

TITLE OF INVENTION

IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT

APPLICANT(S) FOR DO/EO/US

Christine LIBON, Nathalie CORVAIA, Alain BECK, Jean-Yves BONNEFOY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
9. ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Sequence listing paper copy

Sequence listing disk copy

Statement by Attorney under 37 CFR §1.821(f)

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531 PCT

14 SEP 2001

21. The following fees are submitted:

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :**

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =****\$860.00**

Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).

☐ 20 ☐ 30**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	33 - 20 =	13	x \$18.00	<b>\$234.00</b>
Independent claims	1 - 3 =	0	x \$80.00	<b>\$0.00</b>

Multiple Dependent Claims (check if applicable). ☐**\$0.00****TOTAL OF ABOVE CALCULATIONS =****\$1,094.00**Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). ☐**\$0.00****SUBTOTAL =****\$1,094.00**

Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).

☐ 20 ☐ 30

+

**\$0.00****TOTAL NATIONAL FEE =****\$1,094.00**Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐**\$0.00****TOTAL FEES ENCLOSED =****\$1,094.00**

Amount to be:	\$
refunded	
charged	\$

☒ A check in the amount of **1,094** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **8-3220** A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

G. Patrick Sage  
THE FIRM OF HUESCHEN AND SAGE  
500 Columbia Plaza  
350 East Michigan Ave.  
Kalamazoo MI, 49007

SIGNATURE

G. Patrick Sage

NAME

37,710

REGISTRATION NUMBER

September 14, 2001

DATE

PF95PCTSEQ/dln

\* \* \* \* \*

Applicant : Christine LIBON, et al.  
Title : IMMUNOSTIMULANT BACTERIAL MEMBRANE FRAC-  
TIONS IN CANCER TREATMENT

\* \* \* \* \*

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

STATEMENT UNDER 37 CFR 1.821(f)

The undersigned attorney does hereby state that, to the best of his knowledge and understanding, the accompanying Sequence Listing in computer readable form is the same as the accompanying Sequence Listing in paper copy form.

Respectfully submitted,

THE FIRM OF HUESCHEN AND SAGE

By: G. Patrick Sage

G. PATRICK SAGE, #37,710

Dated: September 14, 2001  
Customer No: **25,666**  
500 Columbia Plaza  
350 East Michigan Avenue  
Kalamazoo, MI 49007  
616-382-0030

Enclosure: Sequence Listing in diskette form and paper form

PF95PCTSEQ/dln

\* \* \* \* \*

Applicant : Christine Libon, et al.

Title : IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS  
IN CANCER TREATMENT

\* \* \* \* \*

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231PRELIMINARY AMENDMENT

Sir:

A soon as a Serial Number and Filing Date have been accorded the above-  
identified national phase application, kindly amend as follows:

IN THE CLAIMS: Kindly cancel all of the Claims, 1 through 28, and replace by  
Claims 29 through 61 as provided herewith.

R E M A R K S

The present application is a national phase filing of PCT/FR00/00623.

Applicants have cancelled all of the originally-filed Claims, 1 through 28. New  
Claims 29 through 61 have been added to better encompass the full scope and  
breadth of the invention notwithstanding Applicants' belief that the Claims  
would have been allowable as originally filed. Accordingly, Applicants assert  
that no Claims have been narrowed within the meaning of *Festo*.

Entry of the new Claims and early and favorable action on the merits of this application are respectfully solicited.

Respectfully submitted,

THE FIRM OF HUESCHEN AND SAGE

By:   
G. PATRICK SAGE

Dated: September 12, 2001  
Customer No.: 25,666  
500 Columbia Plaza  
350 East Michigan Ave.  
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Enclosure: Postal Card Receipt  
Claims 29 through 61

We Claim:

- 29 -

The use of a membrane fraction of Gram-negative bacteria, comprising proteoglycans, for preparing a pharmaceutical composition which is immunostimulant and/or which is capable of inducing an antitumor immune response.

- 30 -

The use of Claim 29, wherein the membrane fraction comprises a membrane fraction of *Klebsiella pneumoniae*.

- 31 -

The use of Claim 29, wherein the membrane fraction comprises membrane fractions of at least two different strains of bacteria.

- 32 -

The use of Claim 29, wherein preparation of the membrane fraction comprises the following steps:

- a) culturing the bacteria in a culture medium which allows their growth, followed by centrifugation of the culture;
- b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
- c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;
- d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;
- e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
- f) ultrasonication of the pellet obtained in step e).

- 33 -

The use of Claim 29, wherein preparation of the membrane fraction comprises the following steps:

- a) culturing of the bacteria in a culture medium which allows their growth followed, where appropriate, by centrifugation;
- b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
- c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
- d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
- e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
- f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
- g) sterilization of the concentrated membrane suspension obtained in step f).

- 34 -

The use of Claim 29, wherein the pharmaceutical composition also comprises a vehicle agent for the membrane fraction in a form which makes it possible to improve its stability and/or its immunostimulant activity and/or its capacity to induce an antitumor immune response.

- 35 -

The use of Claim 34, wherein the agent is of the oil-in-water or water-in-oil emulsion type.

- 36 -

The use of Claim 34, wherein the agent is in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.

- 37 -

The use of Claim 29, wherein the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

- 38 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cytokine.

- 39 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from hormones.

- 40 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from growth factors.



- 41 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cellular compound.

- 42 -

The use of Claim 41, wherein the cellular compound is a nucleic acid chosen from DNAs and RNAs.

- 43 -

The use of Claim 41, wherein the cellular compound is a compound of the ribosome family.

- 44 -

The use of Claim 41, wherein the cellular compound is a protein of the heat-shock protein family.

- 45 -

The use of Claim 29 for preparing pharmaceutical composition intended to be administered in combination with an anticancer treatment.

- 46 -

The use of Claim 45, wherein the anticancer treatment is chemotherapy and/or radiotherapy.

- 47 -

The use of Claim 45 for preparing a pharmaceutical composition intended to be administered simultaneously with, separately from, or at intervals with, the anticancer treatment.

- 48 -

The use of Claim 47, wherein the pharmaceutical composition is administered enterally or parenterally.

- 49 -

The use of Claim 45, wherein the combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.

- 50 -

The use of Claim 29 for preventing and/or treating cancers.

- 51 -

The use of Claim 50 for preventing and/or treating bladder cancers, prostate cancers, colon cancers, liver cancers and malignant melanomas.

- 52 -

A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction of Claim 32.

- 53 -

A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction of Claim 33.

- 54 -

The pharmaceutical composition of Claim 52, wherein the Gram-negative bacterium is *Klebsiella pneumoniae*.

- 55 -

The pharmaceutical composition of Claim 53, wherein the Gram-negative bacterium is *Klebsiella pneumoniae*.

- 56 -

The pharmaceutical composition of Claim 52 which is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

- 57 -

The pharmaceutical composition of Claim 53 which is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

- 58 -

The pharmaceutical composition of Claim 56 which contains an anticancer compound as a combination product for use which is simultaneous, separate, or at intervals.

- 59 -

The pharmaceutical composition of Claim 57 which contains an anticancer compound as a combination product for use which is simultaneous, separate, or at intervals.

- 60 -

The pharmaceutical composition of Claim 58, wherein the anticancer compound is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

- 61 -

The pharmaceutical composition of Claim 59, wherein the anticancer compound is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

WO 00/54790

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531 Rec'd PC

14 SEP 2001

IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN THE  
TREATMENT OF CANCERS

- 5 The present invention relates to the use of a membrane fraction of Gram-negative bacteria, in particular of *Klebsiella pneumoniae*, for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response and which is intended, in particular, for treating and preventing
- 10 cancers. The invention also comprises methods for preparing said membrane fractions and also pharmaceutical compositions containing them, in particular combined with anticancer compounds.
- 15 The transformation of a normal cell into a malignant cell is the result of many different events which may occur spontaneously, such as mutations or gene rearrangements, or be induced by chemical, physical or viral agents.
- 20 Tumors are infiltrated by immunocompetent cells, in particular lymphocytes, dendritic cells and macrophages.
- 25 Tumor-associated macrophages (TAMs) originate from the blood circulation and are recruited to the tumor site by cytokines. TAMs bind to the tumor cells via glycoproteins, sugars and phospholipids and proliferate at the tumor site (J. Natl. Cancer Inst., 1998,
- 30 90:1583). There, they secrete many cytokines which contribute to their antitumor activity. Among the most important are TNF- $\alpha$  and IL-12.

- 35 The antitumor activity of TNF- $\alpha$  has been demonstrated in experimental models in mice (Beyaert R. and Fiers W., Cytokines, chapter 24, 335-360 Academic Press. 1998) and has been tested in humans for treating bladder cancers: alone, it has moderate activity

(Steinberg et al., Ann. Oncol., 1992, 3,741-745; Eur. Urol. 1992, 22:112).

5 The production of IL-12 by activated macrophages serves to modulate the immune response by promoting the formation of Th1-type CD4+ T lymphocytes which produce IL-2 and IFN- $\gamma$ . The inhibitory activity of IL-12 on angiogenesis and tumor regression is well known and appears to be linked to the induction of IFN- $\gamma$ , which stimulates the production of IP-10 (interferon-inducible protein-10) and of MIG (monokine induced by IFN- $\gamma$ ) (J. Natl. Cancer Inst., 1998, 90:1583).

15 BCG (Bacille Calmette Guérin) therapy is used to prevent the recurrence of certain types of bladder cancer. The mechanism of action currently proposed is based on the production of cytokines: early release of inflammatory cytokines (TNF- $\alpha$ , IL-1, IL-6, IL-8) and, secondly, production of IL-2 and of IFN- $\gamma$  (Th1 response), then later of IL-4, of IL-5 and of IL-10 (Th2 response). Finally, there occurs a phase of cell activation with amplification of cytotoxic populations (Patard et al., Progrès en Urologie, 1998, 8,415-421).

25 However, BCG therapy does not only have advantages, since the effectiveness sometimes observed is at the expense of a morbidity which is also greater. In addition, there are contraindications for BCG therapy: active tuberculosis (but not prior tuberculosis), immunosuppression (HIV, transplantation, etc.), prior systemic reaction to BCG (hepatitis, pneumonia, BCGitis), steroid treatments. Furthermore, resistances or recurrences exist after BCG therapy.

35 The membrane fraction of K. pneumoniae I145 goes into the composition of a pharmaceutical preparation which prevents the occurrence and recurrence of respiratory infections of bacterial origin and which has been used in humans for 20 years. For this reason, there has been

enough time to assess the nontoxicity of the product. The set of data cited above shows that there exists, today, a need to have novel immunostimulants free of toxic activity. Such immunostimulants would be of great value for treating certain types of cancer.

Surprisingly, the authors of the present invention have demonstrated that membrane fractions of a Gram-negative bacterium, especially *Klebsiella pneumoniae* (named FMKp), in particular membrane fractions obtained using the methods as described hereinafter in the examples, have the desired immunostimulant properties.

The inventors have shown, surprisingly, that the FMKp or one of its major constituents, the OmpA outer membrane protein named P40 (as described in patent applications WO 95/27787 and WO 96/14415) is capable not only of stimulating the proliferation of human blood mononucleated cells, thus demonstrating its immunostimulant activity, but also of inducing, in particular by monocytes, the production of TNF- $\alpha$  and of IL-12, which are cytokines involved in the antitumor immune response.

Thus, the subject of the present invention is the use of a membrane fraction of Gram-negative bacteria, in particular of *Klebsiella pneumoniae*, as a compound which is immunostimulant and/or capable of inducing an antitumor immune response, or for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response, this being whatever the mode of administration in vivo chosen (enteral or parenteral route).

In the present invention, the term "immunostimulant compound" or "immunostimulant pharmaceutical composition" is intended to denote a compound, or a

pharmaceutical composition, capable of increasing a nonspecific immune response.

10 5 In the present invention, the expression "compound capable of inducing an antitumor immune response" or "pharmaceutical composition capable of inducing an antitumor immune response" is intended to denote a compound, or a pharmaceutical composition, capable, in particular, of increasing the effectiveness of an anticancer compound or increasing the effectiveness of an anticancer treatment, such as for example treatment by radiotherapy.

15 The invention also relates to the use as claimed in the invention, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.

20 In the present invention, the expression "membrane fraction of a bacterium" is intended to denote any purified or partially purified membrane fraction or extract which is obtained from a culture of said bacterium and for which the method of preparation comprises at least one step for lysing the bacteria  
25 obtained after culturing and one step for separating the fraction containing the membranes of said bacteria from the total lysate obtained after the lysis step, in particular by centrifugation or filtration.

30 In the present invention, the expression "membrane fraction of a bacterium when said bacterium is *Klebsiella pneumoniae*" is also intended to denote the P40 protein, which is the active fraction of the membrane fraction of *Klebsiella pneumoniae*, of amino  
35 acid sequence SEQ ID No. 2, or a fragment thereof.

According to the invention, the membrane fractions may be prepared according to the methods known to those skilled in the art, such as for example the method

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described by Haeuw J.F. et al. (Eur. J. Biochem, 255, 446-454, 1998).

According to one particular embodiment, the invention  
5 relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

- 10 a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
- b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
- 15 c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;
- d) digestion of the membrane pellet obtained in step c) 20 in the presence of proteolytic enzymes, followed by centrifugation;
- e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
- 25 f) ultrasonication of the pellet obtained in step e).

Step b) for deactivating the lytic enzymes of the bacterial pellet obtained in step a) may be carried out using any known method for deactivating enzymes, such  
30 as, in particular, by heating the resuspended bacterial pellet to a temperature preferably close to 100°C, or by adding an inhibitor of the activity of these enzymes.

35 Step c) for extracting and eliminating the non-membrane-bound proteins and the nucleic acids of the pellet obtained in step a) or b) may be carried out, for example, with at least one cycle of washing the pellet in an extraction solution corresponding to the

addition of a hypertonic solution (extraction solution), preferably a saline solution with a molarity close to 1 M, followed, after a period of contact sufficient for the desired effect, by centrifugation of  
5 the suspension obtained and elimination of the supernatant obtained after said centrifugation, this washing cycle possibly being reproduced several times.

Step d) for digesting the membrane pellet obtained in  
10 step c) may be carried out in the presence of a solution of proteolytic enzymes, such as for example trypsin, chymotrypsin or any known enzyme with proteolytic activity, the conditions of the reaction, pH of the solution, and temperature and duration of the  
15 reaction preferably being adjusted to the optimal conditions for the activity of the enzyme(s) chosen, followed by a centrifugation, this digestion cycle possibly being reproduced several times with the same enzyme or the same combination of enzymes, or with a  
20 different enzyme for each digestion cycle carried out.

Step e) for washing the pellet obtained in step d) is carried out by taking the pellet up in a physiological solution or in distilled water, followed, after a  
25 sufficient period of contact, by a centrifugation, this washing cycle possibly being reproduced several times.

Finally, the objective of step f) for ultrasonication the pellet is, in particular, to disintegrate and  
30 homogenize the membrane fraction obtained at the end of step e). The ultrasonication conditions (duration and power) will be determined by those skilled in the art depending, for example, on the amount of membrane fraction to be treated.

35

According to another particular embodiment, the invention relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

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- 5 a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
- 10 b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
- c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
- 15 d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
- e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
- 20 f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
- g) sterilization of the concentrated membrane suspension obtained in step f).

The conditions for freezing in step b) of the method below will, of course, be determined by those skilled in the art depending on the initial amount of pellet to be treated, preferably carried out at 4°C for at least 48 hours for the equivalent of 1 kg of dried cells.

In step c), the nucleic acids are eliminated, for example, by adding a DNase at a final concentration of 5 mg/ml of a suspension of cells at a concentration equivalent to 5% of dried cells.

The grinding of the cells obtained in step c) may be carried out using any system or apparatus known to those skilled in the art for grinding cells, such as presses or preferably such as Manton Gaulinet loop grinding for 30 minutes.

The clarification of the suspension obtained after grinding may be carried out using any system or apparatus known to those skilled in the art for clarifying ground bacterial cell material, such as the  
5 Sharpless system.

Step e) for precipitating, in acid medium, the suspension obtained in step d) may be carried out, for example, with acetic acid. The precipitation is  
10 followed by elimination of the pellet using, for example, a system of the Sharpless type and by recovery of the supernatant.

Step f) consists of a step in which the supernatant, obtained after precipitation in acid medium, is  
15 neutralized, diluted, dialyzed and then concentrated.

Finally, the last step consists of a step for sterilizing the membrane fraction concentrate obtained  
20 in the preceding step, for instance by heating at 121°C for approximately 35 minutes, for example.

The invention relates particularly to the use as claimed in the invention, characterized in that the  
25 membrane fraction is the *Klebsiella pneumoniae* P40 protein of sequence SEQ ID No. 2, a fragment thereof or a homologous protein, the sequence of which exhibits a percentage identity of at least 80%, preferably 90%, 95% and 99%, with the sequence SEQ ID No. 2, said  
30 fragments or said homologous protein being capable of inducing immunostimulant and/or antitumor activity.

For the purposes of the present invention, the term "percentage identity", "degree of identity" or "level  
35 of identity" between two nucleic acid or amino acid sequences is intended to denote a percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage

5  
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20

25  
30  
35

For example, use may be made of the BLAST program, "BLAST 2 sequences", which is available on the site

<http://www.ncbi.nlm.nih.gov/gorf/bls.html>, the parameters used being those given by default (in particular, for the "open gap penalty" parameter :5 and the "extension gap penalty" parameter :2; the matrix  
5 chosen being, for example, the "BLOSUM 62" matrix proposed by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

10 The expression "fragment of P40 protein" is intended to denote, in particular, any fragment of amino acid sequence included in the amino acid sequence of the P40 protein, which is capable of increasing a nonspecific immune response and/or capable of inducing an antitumor  
15 immune response, and which comprises at least 5 amino acids, preferably at least 10 amino acids, or more preferably at least 15 amino acids.

Of course, said P40 protein, or fragments thereof, may  
20 be obtained by chemical synthesis or in the form of recombinant peptides.

The methods for preparing recombinant peptides are, today, well known to those skilled in the art and will  
25 not be developed in the present description. Among the cells which may be used for producing these recombinant peptides, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli.  
30 Curr. Op. Biotechnology 4:520-525), but also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures (Edwards C.P. and  
35 Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4, 558-563), but also insect cells in which methods implementing, for example, baculoviruses may be used (Luckow V.A., 1993, Baculovirus systems for the

expression of human gene products. Curr. Op. Biotechnology 4, 564-572).

5 A subject of the invention is also the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for vehiculing said membrane fraction in a form which makes it possible to improve its stability and/or its immunostimulant activity and/or its capacity to induce  
10 an antitumor immune response, such as in the form of an emulsion of the oil-in-water or water-in-oil type, or in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and  
15 presented in particulate form.

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for  
20 potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

Among said agents for potentiating the immunostimulant activity and/or the antitumor immune response of said  
25 membrane fractions, cytokines and cellular compounds are preferred.

Among cytokines, mention may be made, without being limited thereto, of: IL-2, IL-12, IL-18, IFN- $\gamma$  and IFN- $\alpha$ .  
30

Among cellular compounds, nucleic acids, compounds of the ribosome family or proteins of the heat-shock protein family are in particular preferred.  
35

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises a potentiating agent which makes it possible to regulate

Among said potentiating agents which make it possible to regulate the immunostimulant activity and/or the antitumor immune response of said membrane fractions, hormones and growth factors are preferred.

Among growth factors, mention may be made, but without being limited thereto, of: EGF, IGF-1, IGF-2, GM-CSF and G-CSF.

According to the invention, the preparation of the pharmaceutical composition is intended to be 25 administered via the enteral or parenteral route, and simultaneously with, separately from or spread out over time with the anticancer treatment.

Many compounds with anticancer activity may thus be  
35 combined with said membrane fraction which is  
immunostimulant and/or capable of inducing an antitumor  
immune response.



Among these compounds, mention may in particular be made, but without being limited thereto, of protease inhibitors or compounds with anti-angiogenic activity, such as for example:

- 5    - protease inhibitors such as TIMPs;  
or the following compounds with anti-angiogenic activity: angiostatin, endostatin, MCP-1, IP-10 and PF-4, and also anti-VEGF, anti-angiogenin, anti-aFGF and anti-bFGF antibodies, antisense sequences or peptides.

10   Thus, the invention relates to the use as claimed in the invention, characterized in that said combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with  
15   anti-angiogenic activity.

The subject of the invention is also the use as claimed in the invention, for preparing a pharmaceutical composition intended to prevent or treat cancers, in  
20   particular bladder cancers, prostate cancers, colon cancers, liver cancers or malignant melanomas.

In another aspect, the invention relates to a method for preparing a membrane fraction of Gram-negative  
25   bacteria, in particular *Klebsiella pneumoniae*, characterized in that it comprises the following steps:

- a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of  
30   said culture;  
b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;  
c) extraction and elimination of the non-membrane-bound  
35   proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;

- d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;
- e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
- f) ultrasonication of the pellet obtained in step e).

The invention also comprises the method for preparing a membrane fraction of Gram-negative bacteria, in particular *Klebsiella pneumoniae*, characterized in that it comprises the following steps:

- a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
- b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
- c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
- d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
- e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
- f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
- g) sterilization of the concentrated membrane suspension obtained in step f).

The membrane fractions which can be obtained using said methods of course form part of the invention.

The titer of proteoglycan of the membrane fractions which can be obtained using said methods, which proteoglycan is the active principle of the FMKp, which

titer is represented by the sum of the galactose and protein contents, is preferably:

- for the galactose : between 1.2 g/l and 3.4 g/l;
- for the proteins : between 7.5 g/l and 14.9 g/l.

5

More preferably, this titer will be:

- for the galactose : between 1.8 g/l and 2.6 g/l;
- for the proteins : between 9.3 g/l and 11.7 g/l.

10 The invention also relates to the pharmaceutical compositions comprising a membrane fraction which can be obtained using the methods as claimed in the invention.

15 Also included in the present invention are the pharmaceutical compositions comprising a membrane fraction of a Gram-negative bacterium, in particular of *Klebsiella pneumoniae*, characterized in that it is combined with an anticancer treatment by chemotherapy  
20 and/or by radiotherapy.

The term "membrane fraction" is herein intended to denote any membrane fraction of the Gram-negative bacterium as defined above, including that which can be  
25 obtained using the methods as claimed in the invention and the P40 protein or a fragment thereof.

Preferably, the invention relates to a pharmaceutical composition as claimed in the invention, characterized  
30 in that it contains an anticancer compound as a combination product for use which is simultaneous, separate or spread out over time, in particular an anticancer compound chosen from protease inhibitors or from compounds having anti-angiogenic activity.

35

Preferably, said pharmaceutical compositions as claimed in the invention may also comprise agents such as vehicles, agents capable of potentiating and/or of regulating the immunostimulant activity and/or the

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antitumor immune response of said membrane fractions as defined above.

The legends to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legends to the figures:

- 10 Figure 1 : Proliferation of PBMC in the presence of FMKp - Dose-response study

The mononucleated cells (PBMC) are obtained by separation with the aid of a solution of Ficoll-sodium metrizoate, using total blood. The PBMC are then seeded in a proportion of 10 000 cells/well in the presence of stimulating agents, in a total volume of 200  $\mu$ l. After incubation for 72 h, the proliferation is objectified by adding tritiated thymidine. The results are expressed as stimulation index = [cpm PBMC + stimulus]/[cpm PBMC without stimulus (= RPMI medium + 10% SVF)].

- 25 Figure 2 : Proliferation of PBMC in the presence of FMKp - Reproducibility of the effect on several donors (FMKp at 250  $\mu$ g/ml).

Figure 3 : Production of TNF- $\alpha$  by blood monocytes

- The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO<sub>2</sub>. Culture conditions : 200 000 cells/well, incubation for 18 h. After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA (Enzyme-Linked ImmunoSorbent Assay) : Predicta kit from Genzyme (detection threshold at 3 pg/ml).

Figure 4 : Production of IL-12 p70 (biologically active) by blood monocytes.

- 5 The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO<sub>2</sub>. Culture conditions : 500 000 cells/well, incubation for 24 h.
- 10 After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA : Endogen antibody pair (detection threshold at
- 15 15 pg/ml).

**Example 1 : Production of the membrane fraction of *K.pneumoniae* (FMKp)**

Method No. 1

- 20 The extraction of the *K. pneumoniae* I145 membranes from the centrifugation pellet from the step is preferably preceded by a step for destroying the lytic enzymes of the cellular components contained in the pellet, for
- 25 example by heating the pellet to 100°C, optionally after redissolving it.

- The actual extraction of the membranes from the centrifugation pellet is preferably carried out by
- 30 treating the cellular components of the pellet, after optional destruction of the lytic enzymes, with a saline solution, for example 1 M sodium chloride, one or more times, then centrifuging the suspension obtained, preferably at 20 000 g; the supernatant from
- 35 this centrifugation, which is eliminated, contains the nonmembrane impurities such as proteins and nucleic acids, while the pellet contains the membranes.

After separation of the saline solution containing the impurities, the membranes are digested in the presence of proteolytic enzymes, preferably trypsin and chymotrypsin, in solution at pH 8, at 37°C for 4 hours.

5

After digestion, the solution is homogenized by ultrasonication. The product thus obtained constitutes the membrane fraction named FMKp.

- 10 The supernatant obtained is centrifuged again under the same conditions, preferably at 140 000 g.

#### **Preparation of the membrane-bound glycopeptides**

- 15 This fraction is prepared from the pellet obtained by centrifugation at 40 000 g for 20 minutes. Said pellet is resuspended in physiological saline and then this suspension is brought to 100°C for 10 minutes in a waterbath of boiling water so as to deactivate the lytic enzymes. After cooling, the suspension is
- 20 centrifuged for 30 min at 20 000 g. The pellet obtained is extracted twice with 1M NaCl in order to eliminate the proteins and the nucleic acids. The membranes are recovered by centrifugation for 30 minutes at 20 000 g.

- 25 They are then subjected to digestion by trypsin at pH 8 and at 37°C for 4 hours, then by chymotrypsin under the same conditions.

- The membranes are then recovered by centrifugation at
- 30 2 000 g for 30 minutes, washed with physiological saline and then distilled water and subjected to 15-minute disintegration by ultrasound.

#### Method No. 2

- 35 After thawing at +4°C for a minimum of 48 h, 1 kg of dry *K. pneumoniae* cells is resuspended at 5% dry cells. DNase is added at 5 mg/l. Next, Manton Gaulin loop grinding is carried out for 30 min, followed by a clarification of a Sharples at 50 l/h, and then

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precipitation with acetic acid at pH = 4.2 + 0.1 for 30 min. The pellet is eliminated (Sharples at 25 l/h) and the supernatant is neutralized and diluted to twice the initial volume with osmosed water. Dialysis at constant volume is then performed on PUF 100 up to 800  $\Omega$ cm, followed by concentration of the membrane suspension (MS) thus obtained, to 11 l/kg of dry cells. The MS is then autoclaved at +121°C for 35 min and can be stored at +4°C for 6 weeks.

#### **Characteristics of the FMKp**

By definition, the titer of proteoglycan, which is the active principle of the FMKp, is equal to the sum of the galactose and protein contents.

- galactose : on average 2.2 g/l
- proteins : on average 10.5 g/l

#### **Example 2 : Proliferation of PBMC from human blood**

The results obtained show that, surprisingly, the FMKp triggers PBMC proliferation. This effect is dose-dependent and maximal for 2.5 mg/ml of FMKp (Figure 1). Moreover, this effect is reproducible (Figure 2).

#### **Example 3 : Production of cytokines by monocytes purified from human blood**

Human monocytes are obtained from the mononucleated cells (lymphocytes, monocytes, NK cells, etc.) isolated beforehand from total human blood. The production of monocytes is based on the expression, in large amount, of the CD14 surface antigen on the cells. The separation is a positive selection. The effectiveness of the magnetic separation of the monocytes is then evaluated by flow cytometry, labeling with a fluorescein isothiocyanate (FITC) - coupled CD13 antibody: the cell suspension then contains 94 to 97% of monocytes.

The results from in vitro studies demonstrate that, interestingly, the FMKp is an immunostimulant which

induces the proliferation of PBMC from human blood with a direct effect on the monocytes : production of TNF- $\alpha$  (Figure 3) and of IL-12 p70 (Figure 4). It is noteworthy that the recombinant P40 protein (rP40), the  
5 OmpA of *K. pneumoniae*, is also capable of stimulating the production of TNF- $\alpha$  (Figure 3) and of IL-12 p70 (Figure 4) by human monocytes.

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CLAIMS

- 1/ The use of a membrane fraction of Gram-negative bacteria, comprising proteoglycans, for preparing a pharmaceutical composition which is immunostimulant and/or which is capable of inducing an antitumor immune response.
- 2/ The use as claimed in claim 1, characterized in that the membrane fraction comprises a membrane fraction of *Klebsiella pneumoniae*.
- 3/ The use as claimed in claim 1 or 2, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.
- 4/ The use as claimed in one of claims 1 to 3, characterized in that the membrane fraction is prepared using a method comprising the following steps:
- a) culturing said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
  - b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
  - c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;
  - d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;

- e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
  - f) ultrasonication of the pellet obtained in step e).
- 5/ The use as claimed in one of claims 1 to 3, characterized in that the membrane fraction is prepared using a method comprising the following steps:
- a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
  - b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
  - c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
  - d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
  - e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
  - f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
  - g) sterilization of the concentrated membrane suspension obtained in step f).
- 6/ The use as claimed in one of claims 1 to 5, characterized in that the pharmaceutical composition also comprises an agent for vehiculing said membrane fraction in a form which makes it possible to improve its stability and/or its

immunostimulant activity and/or its capacity to induce an antitumor immune response.

- 7/ The use as claimed in claim 6, characterized in that said agent is of the oil-in-water or water-in-oil emulsion type.
- 8/ The use as claimed in claim 6, characterized in that said agent is in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.
- 9/ The use as claimed in one of claims 1 to 8, characterized in that the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.
- 10/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cytokine.
- 11/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from hormones.
- 12/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from growth factors.

- 13/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cellular compound.
- 14/ The use as claimed in claim 13, characterized in that said cellular compound is a nucleic acid chosen from DNAs and RNAs.
- 15/ The use as claimed in claim 13, characterized in that said cellular compound is a compound of the ribosome family.
- 16/ The use as claimed in claim 13, characterized in that said cellular compound is a protein of the heat-shock protein family.
- 17/ The use as claimed in one of claims 1 to 16, for preparing a pharmaceutical composition intended to be administered in combination with an anticancer treatment.
- 18/ The use as claimed in claim 17, characterized in that the anticancer treatment is chemotherapy and/or radiotherapy.
- 19/ The use as claimed in either of claims 17 and 18, for preparing a pharmaceutical composition intended to be administered simultaneously with, separately from or spread out over time with the anticancer treatment.
- 20/ The use as claimed in claim 19, characterized in that the pharmaceutical composition is administered via the enteral or parenteral route.

- 21/ The use as claimed in one of claims 17 to 20, characterized in that said combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.
- 22/ The use as claimed in one of claims 1 to 21, for preventing and/or treating cancers.
- 23/ The use as claimed in claim 22, for preventing and/or treating bladder cancers, prostate cancers, colon cancers, liver cancers and malignant melanomas.
- 24/ A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction as described in claim 4 or 5.
- 25/ The pharmaceutical composition as claimed in claim 24, characterized in that said Gram-negative bacterium is *Klebsiella pneumoniae*.
- 26/ The pharmaceutical composition as claimed in claim 24 or 25, characterized in that it is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.
- 27/ The pharmaceutical composition as claimed in claim 26, characterized in that it contains an anticancer compound as a combination product for use which is simultaneous, separate or spread out over time.
- 28/ The pharmaceutical composition as claimed in claim 27, characterized in that said anticancer compound

is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

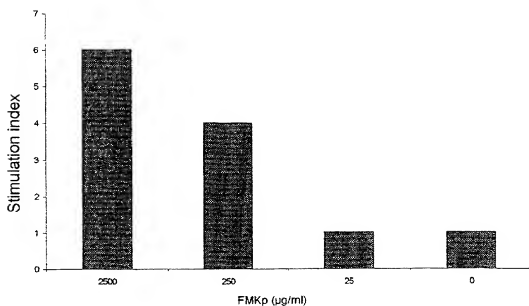


FIGURE 1

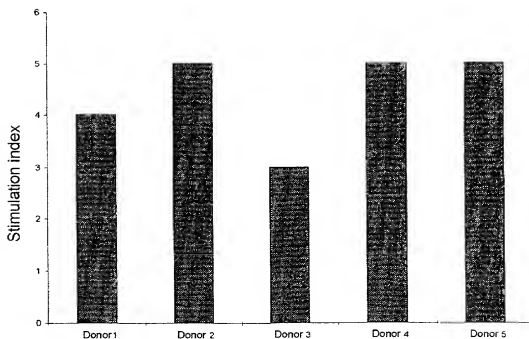


FIGURE 2

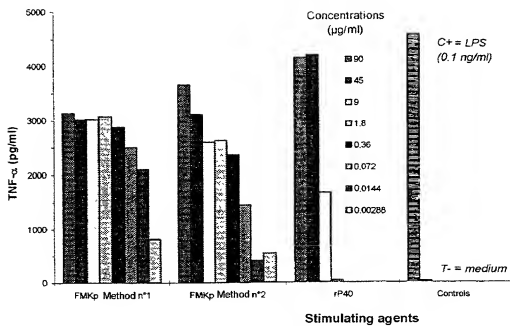


FIGURE 3

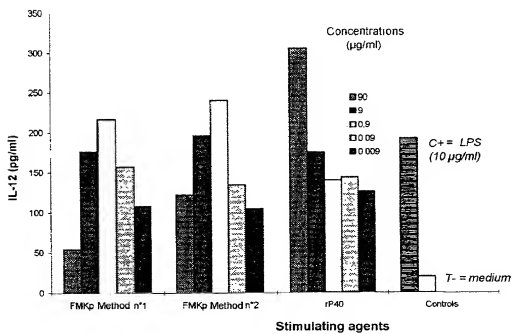


FIGURE 4



**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, next to my name, and

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER**

**TREATMENT**

the specification of which (check one of the following)

☒ is attached hereto

was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

And was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor(s) certificate having a filing date before that of the application on which priority is claimed:

<u>Application Serial Number</u>	<u>Country</u>	<u>Filing Date (Day/Month/Year)</u>	<u>Priority Claimed (yes/no)</u>
99 03154 ✓	FRANCE ✓	15/March/1999 ✓	Yes

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Docket Number: \_\_\_\_\_

(Application Serial No.)  
**PCT/FR00/00623** ✓

(Filing Date)  
**15.03.2000** ✓

(Status ~~XXXXXX~~, pending ~~XXXXXX~~)

(Application Serial No.)

(Filing Date)

(Status - patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status - patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following person registered to practice before the Patent and Trademark Office as my attorney with full power of substitution and revocation to prosecute this application and all divisions and continuations thereof and to transact all business in the Patent and Trademark Office connected therewith and request that all correspondence be sent to him at the mailing address hereafter given:

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G. PATRICK SAGE



25666

PATENT-TRADEMARK OFFICE

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Name

Telephone

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Inventor's Signature:

Date: **2.07.2001**


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Date:

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Citizenship:  
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Inventor's Signature:

Date:

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Citizenship:  
(Country)

Post Office Address:

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<110> LIBON Christine  
CORVAIA Nathalie  
BECK Alain  
BONNEFOY Jean-Yves

<120> IMMUNOSTIMULATING BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT

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## SEQUENCE LISTING

<110> PIERRE FABRE MEDICAMENT

<120> USE OF BACTERIAL MEMBRANE FRACTIONS WITH  
IMMUNOSTIMULANT ACTIVITY IN THE TREATMENT OF  
CANCERS, METHODS FOR PREPARING THEM AND THE  
PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

<130> D17974

<140> FR 99 03154

&lt;141&gt; 1999-03-15

&lt;160&gt; 4

&lt;170&gt; PatentIn Vers. 2.0

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